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TITLE: Understanding the link between pregnancy and breast cancer.

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13. SUPPLEMENTARY NOTES

14. ABSTRACT Our study is testing whether pregnancy affect the growth of breast cancer cells that expressed a mutant form of p53. We found that the growth of genetically identical cells carrying a mutation in p53 is accelerated by the passage through pregnancy. The tumors volumes are bigger in the pregnancy group compared to the virgin group. We also found using microarray analysis that there are genes that are up-regulated specifically in the pregnancy group and not in the virgin group. We also obtained mice carrying a p53 mutant knockin in the p53 knockout mice background. We have established a colony and we currently have females that have been through 1, 2 or 3 pregnancies. These mice will be analyzed for mammary tumor formation. We also have established transgenic mice expressing PAPP-A specifically in their mammary glands. We recently found that these mice do express PAPP-A and are currently establish a colony for analysis. In addition, we have now collected the blood and primary breast cancer sections of 81 breast cancer patients prior to their surgery.

15. SUBJECT TERMS Pregnancy-associated breast cancer, pregnancy-associated plasma protein A, p53.					
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Table of Contents

INTRODUCTION:

The subject of this research is to understand the link between pregnancy and breast cancer. Pregnancy is associated with an immediate but transient increased risk of breast cancer in all women, with the risk peaking within 5 years after pregnancy. Breast cancer arising shortly after pregnancy are most frequently triple-negative breast cancers, which are characterized by a high rate of p53 mutation and by the activation of IGF signaling. The purpose of this research is to begin to understand the role of pregnancy in breast cancer by focusing on the p53-PAPPA-IGFII axis we have discovered and to test our hypothesis that Pregnancy-associated plasma protein A (PAPPA), a protease required for the activation of IGF signaling, is involved.

The scope of this research is 1) to monitor the activation of IGF signaling and test the efficacy of anti-IGF therapy in our established triple-negative cell line model of pregnancy-dependent breast cancers, 2) to dissect the $p53^{MUT}$-PAPP-A axis in pregnancy-associated mammary and 3) to determine whether PAPP-A expressing breast cancers are associated with increased serum levels of PAPP-A and IGF-II.

BODY:

Task 1: Establish $p53^{WT}$ and $p53^{MUT}$ xenografts from virgin and parous females.

Research accomplishments:

We have created $p53^{MUT}$ xenografts from virgin and parous females. MDA-MB231 cells were injected in the mammary glands of nude mice females and 2 groups were generated virgin group and the pregnant group, in which a male was added for mating. Pregnancies were recorded. Immediately after pregnancy, the pups were removed to allow involution of the mammary glands. Tumor formation was monitored weekly and tumor volumes determined. For the tumor bank, the tissue was immediately frozen in freezing medium to conserve viability. Another part was fixed in 10% formalin and analyzed for histology and yet another part was preserved for Western or qRT-PCR analyses. For the latter, the mammary gland tissue was be snap frozen in liquid nitrogen and pulverized into a powder that can then be used either for protein or RNA extraction. The levels of IGF-II and PAPP-A was determined by qRT-PCR since the current antibodies do not recognize mouse proteins. We found that the volume of tumors derived from mice that have been through pregnancies was significantly higher than in the virgin groups (Fig. 1).

We also performed analysis of IGFII and PAPP-A but did not see a significant difference between the two groups. However, histological analysis of the tumor revealed a stricking difference. We found that while virgin-derived tumors show a basal-like histology, thoese derived from parous females show a more luminal phenotype (Fig. 2).

Task 2: Establish the molecular signature of the $p53^{WT}$ and $p53^{MUT}$ xenografts from virgin and parous females.

Research accomplishments:

We have performed microarray analysis of the tumor derived from virgin or parous females. RNA was extracted and hierarchical clustering was done using DNA-Chip Analyzer software. This analysis was performed using the Genomics Core facility at Mount Sinai. We found that several genes distinguish the two groups. Our initial analysis revealed at least two signatures that are significantly different between the two groups. These were identified using the Gene pattern program. In the Konco results, the cyclin D1KE upregulated signature showed a FDR value of 0.071. This is of interest since cyclin D1 is essential for prolactin signaling during pregnancy. We also found under the CGP results, the Charafe breast cancer luminal vs basal signature. This is of interest since we do observed a change in histological pattern.

We are currently validating these signatures.

Task 3: Test the sensitivity of $p53^{WT}$ and $p53^{MUT}$ xenografts from virgin and parous females to anti-IGF therapy.

Research accomplishments:

This task has not been started yet

Task 4: Establish the rate of mammary tumor in p53-mutant knock-in mice. In this aim, rate of mammary tumor in the p35MUT knock-in mice will be determined in 4 different groups; 1) virgin, 2) following 1 pregnancy, 3) following 2 pregnancies and 4) following

3 pregnancies. Because p53 KI mice can die of lymphoma, the mammary gland will be extracted and transplant into wild type recipient mice.

Research accomplishments:

The colony has been established. Table 1 show the current status of the colony:

Task 5: Establish and characterize the effect of PAPP-A on the mammary gland.

Research accomplishments:

We have created MMTV-PAPP-A transgenic mice. First, we have determined by both RT-PCR (Fig. 3) and by immunohistochemistry (Fig. 4) that these transgenic mice express PAPP-A.

We are currently building the colony for the analysis of the effect of PAPP-A expression during post-natal development, adult virgin, during pregnancy, during involution as well as in tumor formation following pregnancy. The current status of the coloby is shown in table 2.

Task 6: Collect 300 tissue samples and serum from breast cancer patients to determine the levels of PAPP-A in the serum and in the tumor sections.

Research accomplishments:

We have so far collected blood and primary breast cancer sections from 81breast cancer patients.

We will determine the PAPP-A levels in the serum of these patients. The PAPP-A (R&D systems cat # DPPA00), and IGF-II (DSLabs) Enzyme-Linked ImmunoSorbent Assay (ELISA) are a solid phase assay. We have so far completed the analysis on 11 patients. One of 11 shows a significant elevation in serum (Fig. 5)

For the immunohistochemistry, staining in primary tumor sections will be performed for PAPP-A using the rabbit anti-human PAPP-A antibody at a dilution of 1:125 (Dako Laboratories, Santa Cruz). The scoring of the slides will be performed by our breast pathologist, Dr. Shabnam Jaffer. This analysis will begin shortly.

KEY RESEARCH ACCOMPLISHMENTS:

- We have determined that tumors derived from genetically identical breast cancer cells grow faster following passage through pregnancy. Therefore, pregnancy stimulates the growth of cancer cells.
- We found that several genes are differentially expressed in virgin and parous-derived mammary tumors.
- We have established a p53 knockin mice colony.
- We have established the MMTV-PAPP-A transgenic mice.
- We have collect samples from 81 patients and are currently beginning their analysis.

REPORTABLE OUTCOMES:

Manuscripts, abstracts, presentations: No

Licenses applied for or issued: No

Degrees obtained that are supported by this award: No

Development of cell lines, tissue or serum repositories:
Yes, we have collected tissues and blood from 81 breast cancer patients

Informatics such as databases and animal models:
Yes, we have established a novel animal model (MMTV-PAPP-A) and we are generated microarray data on the pregnancy versus virgin tumors.

Funding applied for based on work supported by this award: No

Employment or research opportunities applied for: No

CONCLUSION:
In conclusion, we have obtained data supporting our original hypothesis that pregnancy does accelerate the growth of cells carrying a p53 mutation. We have also obtained data suggesting that PAPP-A may be an oncogene due to the hyperproliferation of the mammary ducts, although this is preliminary.

So what? The knowledge gained from this information is far reaching since it may led to the design a therapy targeted specifically at breast cancers that arise shortly after pregnancy and are directly linked to the remarkable tissue remodeling of the breast during pregnancy.

References:
Not applicable

Appendices:
None

Supporting Data:

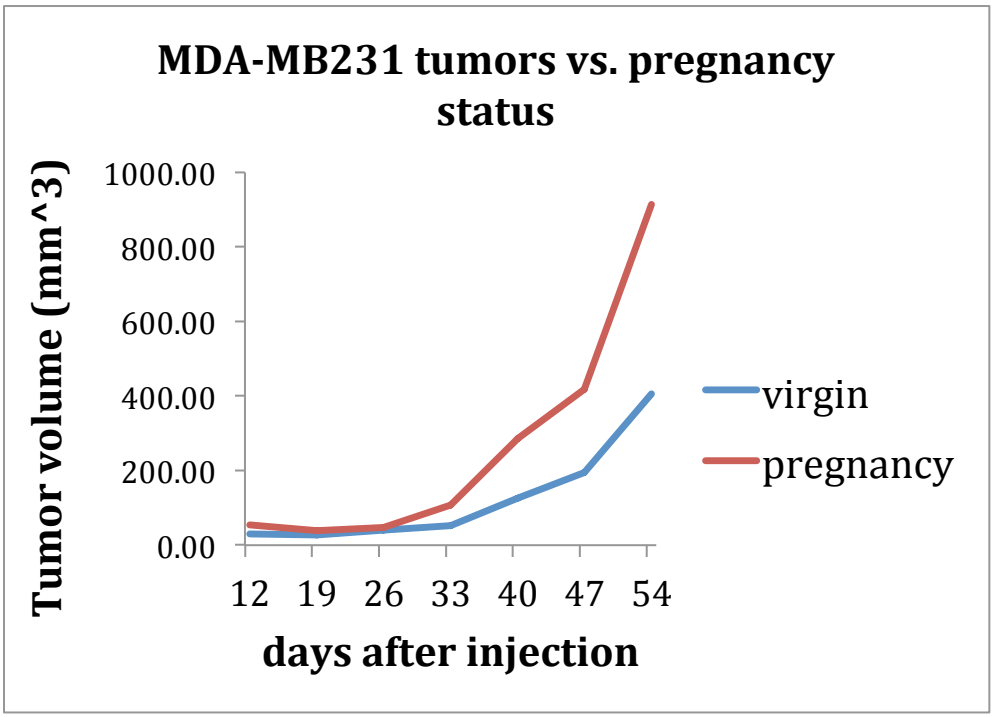

Fig. 1: Growth curve of tumor xenografts derived from virgin or parous females over time is shown.

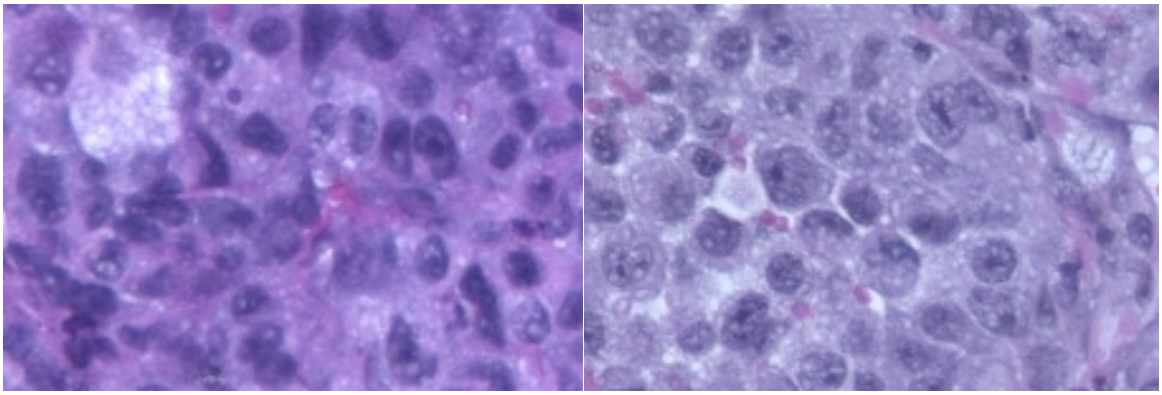

Fig. 2 H&E of MDA-MB231 (mutant for p53) xenografts from virgin or parous females.

Female	Cage	DOB	Age (weeks)	Virgin	# Pregnancies	date of last pregnancy	Group name	Time since last pregnancy (weeks)
1-1ex 1	1A	6-Feb	17	no	on #2	13-Jun	Pregnancy 2 (invol)	0
1-1ex 2	1A	13-Feb	16	no	on #2	13-Jun	Pregnancy 2 (invol)	0
1-1ex 3	1B	13-Feb	16	no	1	3-May	Pregnancy 1	5
1-1 ex 4	1B	22-Feb	15	no	1	3-May	Pregnancy 1	5
1-1 ex pup 1	3	3-May	5	yes			Virgin	
1-1 ex pup 2	3	3-May	5	yes			Virgin	
1-1 ex pup 3	3	3-May	5	yes			Virgin	
1-1 ex pup 4	3	3-May	5	yes			Virgin	
1-1 ex pup 5	4	3-May	5	yes			Virgin	
1-1 ex pup 6	4	3-May	5	yes			Virgin	
1-1 ex pup 7	4	3-May	5	yes			Virgin	
1-1 ex pup 8	4	3-May	5	yes			Virgin	
1-2 ex 1	1A	18-Mar	12	no	on #2	13-Jun	Pregnancy 2 (invol)	0
1-2 ex 2	1B	18-Mar	12	no	1	24-May	Pregnancy 1	2
1 founder	7	?	?	no	3	11-May	Pregnancy 3	4
3 founder	7	?	?	no	3	13-May	Pregnancy 3	4
3-2 1	9	13-May	4	yes			Virgin stock	
3-2 2	9	13-May	4	yes			Virgin stock	
3-2 3	9	13-May	4	yes			Virgin stock	
3-2 4	9	13-May	4	yes			Virgin stock	
3-1 3	1B	3-Apr	9	no	1	10-Jun	Pregnancy 1 (invol)	0
4 founder	7	?	?	no	3	27-Apr	Pregnancy 3	6
4 pups 1	3	27-Apr	5	yes			Virgin	
4 pups 2	4	27-Apr	5	yes			Virgin	
4-1 ex 1	14	4-Mar	13	no	1	24-May	Pregnancy 1-2 (invol)	2
4-1 ex 3	14	4-Mar	13	no	1	1-Jun	Pregnancy 1-2 (invol)	1
4-1 ex 4	14	4-Mar	13	no	1	7-Jun	Pregnancy 1-2 (invol)	0
5 founder	5	?	?	no	2	21-May	Pregnancy 3	2

Table 1: List of current status of the p53 mutant knockin mice colony.

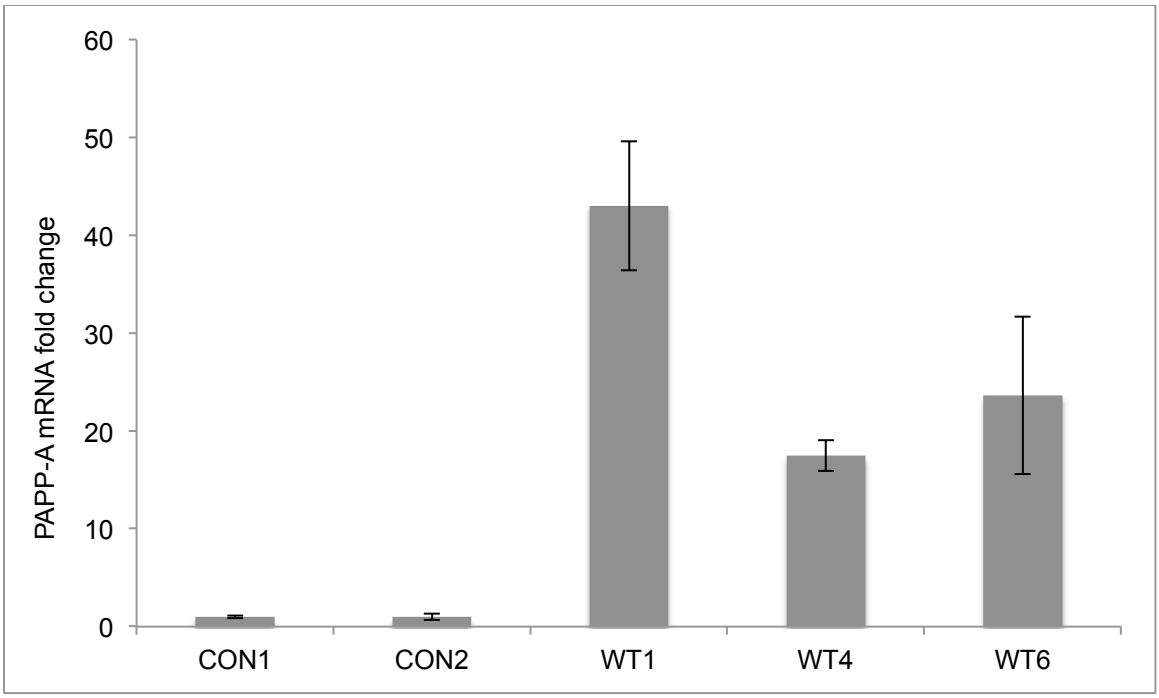

Fig. 3 RT-PCR determination of PAPP-A mRNA levels in controls non-transgenic (CON) and three different founders expressing wild-type PAPPA in the mammary glands.

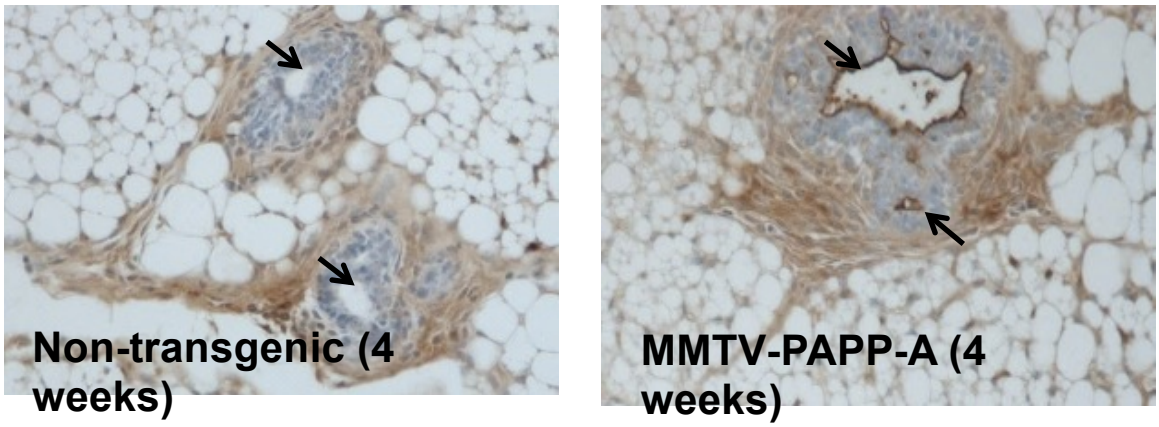

Fig. 4 Immunohistochemistry of PAPP-A in mammary glands of non-transgenic and MMTVPAPP-A transgenice at the age of 4 weeks. Blue indicayes the nucleus of the mammary ducts. Arrows indicate lumen of ducts. Note the stainingin the lumen of PAPP-A transgenic mice.

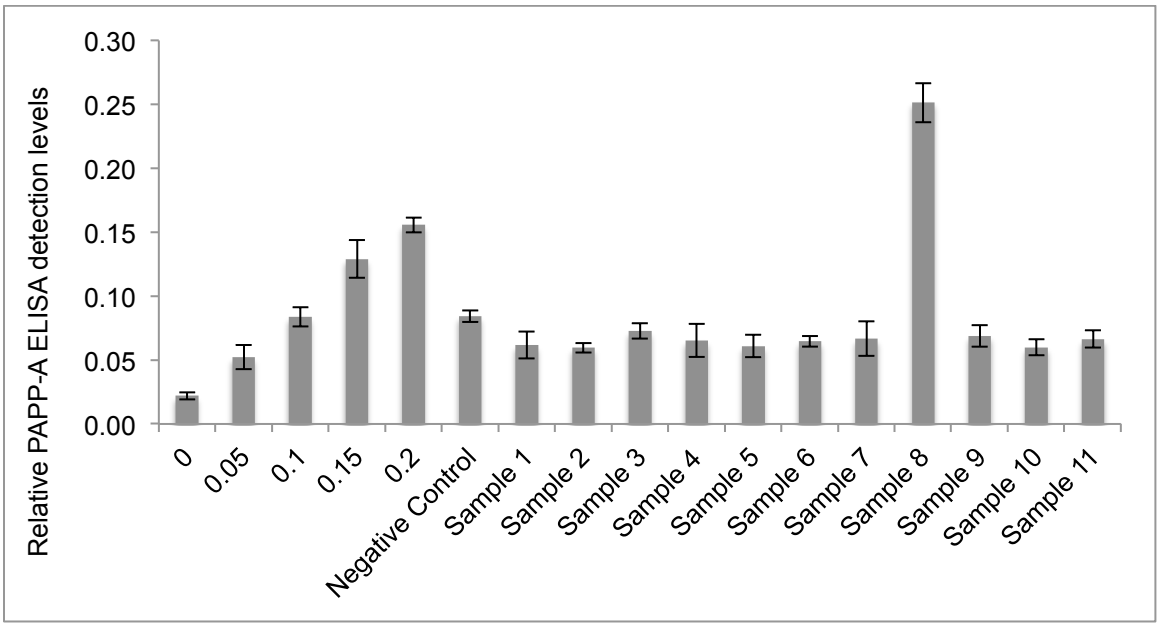

Fig. 5 PAPP-A ELISA assay using standard cruve of recombinant PAPP-A (first 5 lanes) follow by serum from 11 patients.